ATP stimulates the release of prostacyclin from perfused veins isolated from the hamster hindlimb

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Hammer, Leah W., Carmen R. Overstreet, Jaehwa Choi, and Robert L. Hester. ATP stimulates the release of prostacyclin from perfused veins isolated from the hamster hindlimb. Am J Physiol Regul Integr Comp Physiol 285: R193–R199, 2003. First published March 20, 2003; 10.1152/ajpregu.00468.2002.—ATP-stimulated prostacyclin release from veins was investigated using epigastric veins isolated from hamsters. Veins were perfused with MOPS-buffered physiological salt solution (PSS). ATP was administered into the perfusate, and the bath solution (MOPS-PSS) was collected and assayed for the presence of the stable prostacyclin metabolite 6-keto-PGF1α. ATP (100 μM) resulted in reproducible increases in bath concentration from 73 ± 22 to 279 ± 50 pg/ml (P < 0.05, n = 5). This response was abolished by indomethacin (10 μM, P < 0.05). To ascertain whether the endothelium was the source of prostacyclin, endothelium was disrupted using air (n = 10) or deoxycholic acid (n = 6). Perfusion with air significantly reduced (P < 0.05) but did not completely abolish ATP-stimulated release of prostacyclin, while deoxycholic acid totally abolished the response (P < 0.05). The nonselective P2 receptor antagonist reactive blue 2 (100 μM) attenuated ATP-mediated release of prostacyclin but did not significantly alter ACh-stimulated release of prostacyclin. The nonselective adenosine receptor antagonist xanthine amine congener (1 μM) had no effect on ATP-stimulated release, and adenosine did not stimulate the release of prostacyclin. These results show that increases in intraluminal concentration of ATP stimulate abluminal release of prostacyclin from the venous endothelium. This effect is mediated by P2 receptors while adenosine and its receptors are not involved in this response.

prostaglandins; P2Y receptor; cyclooxygenase

A recent study has reported a correlation between venous plasma ATP concentration and the oxygenation state of hemoglobin in humans (12). We have further demonstrated that ATP infused into venules paired with arterioles results in dilation of the adjacent arteriole in hamster cremaster muscle, an effect that was blocked by disruption of the venular endothelium or by global administration of indomethacin (15). The results of some of these studies suggest that during periods of increased skeletal muscle metabolism, such as during exercise, red blood cells release ATP in the venular blood in response to stimuli such as hypoxia, hypercapnia, or decreased pH. ATP then stimulates the venular endothelium to produce prostacyclin, which diffuses to and dilates the adjacent arteriole. We recently demonstrated that increases in intraluminal concentration of ATP stimulated the release of prostacyclin from hamster epigastric veins via an increase in intracellular calcium concentration (5). The first aim of the present study was to determine whether the endothelium is the source of the prostacyclin released in response to ATP in these veins.

Once released from cells, extracellular ATP is rapidly hydrolyzed to ADP, AMP, and adenosine via extracellular ectonucleotidases. The fate of extracellular ATP has been reviewed elsewhere (3, 13, 28). ATP and ADP mediate their vasodilatory effects via P2Y receptors while adenosine and AMP mediate their vasodilatory effects via adenosine (P1) receptors. Subtypes of both receptors have been identified on vascular endothelial cells (22). The relative contribution of P2Y and P1 receptors to ATP-mediated vasodilation remains controversial, and thus a second aim of the present study was to determine whether ATP-stimulated prostacyclin release was mediated via activation of P2Y or P1 receptors.

MATERIALS AND METHODS

The experimental protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center and were carried out according to both the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and the guidelines of the Animal Welfare Act.
General Procedures

Male golden hamsters (140–200 g) were anesthetized with pentobarbital sodium (60 mg/kg ip). The hindlimb vasculature was exposed by an incision of the skin, and the tissues were kept moist by superfusing them with warm physiological salt solution (PSS) of the following composition (in mM): 131.9 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, and 20 NaHCO3, equilibrated with 95% N2-5% CO2. With the use of an operating microscope, the superficial epigastric vein was separated from the adjacent superficial epigastric artery and surrounding tissue, and small branches were ligated using 10–0 nylon suture. Attempts were made to obtain as great a length of vein as possible; however, branching of this vessel into three smaller vessels occurred within 5 mm of its origin at the femoral vein, limiting the length of the vessels in situ to 3–5 mm. Using a catheter containing MOPS-buffered PSS (composition in mM: 145 NaCl, 4.7 KCl, 2.0 CaCl2, 1.17 MgSO4, 1.2 NaH2PO4, 0.02 EDTA, 3.0 MOPS, 5.0 glucose), the vein was cannulated at its proximal end, removed from the animal, and flushed of blood. After transferring the vessel to a 1-ml bath containing warm (37°C) MOPS-PSS, a second catheter was placed in the distal end of the vessel, and perfusion with MOPS-PSS at 37°C, bubbled with CO2, was commenced. Vessels were perfused from a reservoir at a fixed height and constant pressure (20 mmHg static pressure, 1 ml/min). Because the majority of the resistance of this system was in the tubing before the vessel, any changes in vessel diameter, which may have occurred during the experiments, were unlikely to have any significant effect on perfusion pressure and thus were unlikely to be of any functional significance in the present study.

At the completion of the experimental protocols (described below), the vessels were removed from the catheters and fixed with 10% formalin. Vessels were then paraffin embedded, cut into 5-μm sections, and stained with hematoxylin and eosin. Vessels were viewed under a light microscope to confirm the presence of endothelium.

Experimental Protocols

Values of n are the number of vessels used for a given protocol. Different protocols were undertaken when two vessels were harvested from the same animal. Only one protocol was carried out in each vessel. Vessels were equilibrated for 1 h with collection of bath solution commencing at 30 min. The entire 1-ml bath content was collected and replaced with fresh MOPS-PSS every 10 min for the duration of the experiment. Samples were stored at −20°C until they were assayed, in triplicate, for the stable metabolite of prostacyclin, 6-keto-PGF1α, using the Elisa Technologies 6-keto-PGF1α kit (Neogen, Lexington, KY; product 404310). This technique has an assay range of 20–2,000 pg/ml, with an intra- and interassay coefficient of variation of 10%. An increase in the amount of 6-keto-PGF1α, measured in the bath solution was interpreted as an increase in the amount of prostacyclin released from the veins. In all of the following experiments, ATP (100 μM), ACh (10 μM), or adenosine (100 μM) was perfused for 30 min to establish a control response. After a washout period of 10 min, during which time the vessels were perfused with regular MOPS-PSS, the veins were treated with an inhibitor (which remained in the perfusate solution until the end of the experiment) or the endothelium was disrupted using one of two methods described below. Vessels were then exposed to the same agonist used to establish the control response.

Protocol 1: cyclooxygenase inhibition. The effect of cyclooxygenase inhibition on ATP-mediated prostacyclin release was examined by perfusing the vessels with indomethacin (10 μM, n = 6) for 20 min before establishing a second response to ATP. Time and vehicle (sodium carbonate, 10 mM) controls were performed in additional vessels (n = 5 and n = 6, respectively).

Protocol 2: endothelial disruption. The endothelium was disrupted in one of two ways. First, an air bubble was perfused through the vessel for several seconds before establishing the second response to ATP (n = 10). The second protocol utilized the detergent deoxycholic acid. This was backflushed through the vein immediately after the first exposure to ATP (n = 6). Vessels were then allowed to stabilize for 20 min before perfusing with ATP for a second time.

Protocol 3: purine (P2) receptor blockade. To determine whether ATP was mediating its effect via P2 receptors, the nonselective antagonist reactive blue 2 (100 μM, n = 6) was added to the perfusate solution 20 min before the second exposure to ATP. To determine that the effect of this antagonist was specific for ATP, these experiments were repeated in different vessels using ACh in place of ATP (n = 5).

Protocol 4: adenosine receptor blockade. To determine whether the ATP-mediated release of prostacyclin was due to the hydrolysis of ATP to adenosine, experiments were performed whereby adenosine (100 μM) was used instead of ATP. The P1 receptor antagonist xanthine amine congener (XAC, 1 μM) was added to the perfusate before the second exposure to adenosine (n = 5). XAC has been reported to block the A1 and A2 subtypes of the P1 receptor (10). In additional vessels (n = 4), we examined the effect of XAC (1 μM) on bath concentrations of 6-keto-PGF1α, in response to ATP, to determine whether other metabolites of ATP that act at P1 receptors were responsible for prostacyclin release. As with all of the protocols utilizing inhibitors, XAC was added to the perfusate 20 min before the second exposure to ATP and remained in the solution for the duration of the experiment.

Drugs

All drugs were purchased from Sigma (St. Louis, MO). ATP was dissolved in distilled water (100 mM), and XAC was dissolved in DMSO (100 μM). Both drugs were stored in aliquots at −20°C and thawed on the day of the experiment. Indomethacin was dissolved in sodium carbonate (10 mM) and stored in the refrigerator. Adenosine was dissolved and diluted in MOPS-PSS on the day of the experiment, and aliquots of ATP, indomethacin, and XAC were diluted in MOPS-PSS on the day of the experiment. Deoxycholic acid (3%) was suspended in distilled water. On the day of the experiment, the solution was heated to 37°C before back-flushing the vessel.

Analytic and Statistical Methods

As each vessel was exposed to ATP or adenosine before and after an experimental protocol, the effects of ATP and of the experimental protocol on bath concentrations of 6-keto-PGF1α were analyzed using a two-way repeated measures ANOVA. All data are means ± SE. Statistical significance was accepted at P < 0.05.

RESULTS

Figure 1 shows the effect of increasing concentrations of intraluminal administration of ATP on ablu-
minal release of prostacyclin as determined through measurement of bath concentrations of the stable metabolite 6-keto-PGF$_{1\alpha}$. In the absence of ATP, basal levels of 6-keto-PGF$_{1\alpha}$ were detected in the bath solution ($172 \pm 35$ pg/ml, $n = 7$). ATP (1, 10, and 100 μM) resulted in dose-dependent increases in the amount of 6-keto-PGF$_{1\alpha}$ measured ($213 \pm 37, 322 \pm 63, 680 \pm 216$ pg/ml, respectively, $n = 7$); however, due to the large variability in basal levels of release, statistical significance was only achieved at 100 μM. Thus this concentration was used throughout this study. Figure 2A demonstrates that ATP (100 μM)-stimulated release of prostacyclin was reproducible ($n = 5$); however, it is interesting to note that the second challenge to ATP was met with a decreased response if a minimum of 30 min was not allowed between ATP challenges (data not shown). The ATP-stimulated increase in bath concentration of 6-keto-PGF$_{1\alpha}$ was abolished by intraluminal administration of indomethacin (Fig. 2B, $P < 0.05, n = 6$). The vehicle for indomethacin, sodium carbonate, had a small but significant effect on ATP-stimulated release of prostacyclin (Fig. 2C, $P < 0.05, n = 6$).

The effect of endothelial disruption on 6-keto-PGF$_{1\alpha}$ release is shown in Fig. 3. ATP (100 μM) was associated with an increase in 6-keto-PGF$_{1\alpha}$ concentration from $160 \pm 32$ to $653 \pm 139$ pg/ml (Fig. 3A, $P < 0.05, n = 10$). Disruption of the endothelium using air bubbles significantly decreased ATP-mediated prostacyclin release but did not completely abolish this response (from $144 \pm 33$ to $421 \pm 102$ pg/ml, $n = 10$, Fig. 3A). However, when the venous endothelium was disrupted using deoxycholic acid, the response to ATP was totally abolished (Fig. 3B, $P < 0.05, n = 6$).

The nonselective P2 receptor antagonist reactive blue 2 (100 μM) was shown to significantly decrease ATP-stimulated increases in 6-keto-PGF$_{1\alpha}$ concentration (from $537 \pm 111$ to $205 \pm 82$ pg/ml, $P < 0.05, n = 6$, Fig. 4A). In contrast, 100 μM reactive blue 2 was without effect on ACh-stimulated increases in 6-keto-PGF$_{1\alpha}$ concentration (Fig. 4B, $n = 5$).

ATP-stimulated increases in 6-keto-PGF$_{1\alpha}$ were not altered by the adenosine (P1) receptor antagonist XAC (1 μM, $n = 4$, Fig. 5A), and adenosine (100 μM) was not associated with any increase in bath concentration of 6-keto-PGF$_{1\alpha}$, from these veins (Fig. 5B, $n = 5$).

**DISCUSSION**

This study tested the hypothesis that increases in luminal concentrations of ATP can result in the abluminal release of prostacyclin from venous endothelium. Perfusion of epigastric veins, isolated from the hamster hindlimb, with ATP resulted in large increases in bath concentration of the stable prostacyclin metabolite 6-keto-PGF$_{1\alpha}$, indicating large ATP-mediated increases in prostacyclin release from these vessels. The response to ATP was blocked by indomethacin and was partially or totally blocked by damage to the venous endothelium. These results provide direct evidence that an increase in intraluminal ATP concentration...
can stimulate prostacyclin release from endothelial cells situated on the venous side of the circulation.

We also sought to clarify whether ATP was exerting its effect via direct stimulation of purine P2 receptors or whether a metabolite such as adenosine was mediating the effect. The results show that the nonselective P2 antagonist reactive blue 2 did inhibit ATP-mediated prostacyclin release. In addition, the adenosine receptor antagonist XAC had no effect on the response elicited by ATP while direct application of adenosine did not stimulate the release of prostacyclin. These results rule out a role for adenosine receptors in mediating this effect and suggest that ATP is exerting its effect via direct stimulation of P2 receptors on the venular endothelium.

Increases in skeletal muscle metabolism are associated with the release of a cyclooxygenase product from the venular endothelium, which can influence the diameter of the adjacent arteriole.

Fig. 4. Effect of the nonselective P2 receptor antagonist reactive blue 2 on ATP-stimulated (A) and ACh-stimulated (B) 6-keto-PGF1α release. *ATP-mediated release significantly different from basal release. #ATP response after endothelium disruption is significantly different from ATP response before disruption (P < 0.05).

Fig. 5. Effect of the nonselective P1 receptor antagonist xanthine congener (XAC, 1 mM) on ATP (100 μM)-stimulated (A) and adenosine (1 μM)-stimulated (B) 6-keto-PGF1α release. *ATP-mediated release significantly different from basal release (P < 0.05).
In 1995, Ellsworth and colleagues (9) demonstrated that red blood cells could release ATP under hypoxic and low-pH conditions. As such conditions occur in the venous circulation during exercise, we hypothesized that ATP might play a role in functional hyperemia. We (15) have shown that ATP infused into a venule running parallel to an arteriole results in dilation of the cremasteric arteriole. This effect of ATP was shown to be dependent on an intact venular endothelium and was inhibited when indomethacin was applied globally to the entire cremaster preparation. These studies support a role for ATP in control of vascular tone and further support our hypothesis that cyclooxygenase metabolites from the venular endothelium can influence arteriolar diameter. Furthermore, a recent study demonstrated that venous plasma ATP concentration progressively increased with incremental exercise, suggesting that circulating ATP levels were tightly coupled to the oxygenation of hemoglobin in healthy humans (12).

In the present study, we aimed to provide direct evidence for ATP-stimulated release of prostacyclin from veins. Veins were cannulated at both ends and were mounted in a 1-ml chamber containing PSS. This setup allowed us to administer the ATP intraluminally and measure the prostacyclin release abluminally. Addition of ATP to the perfusion solution resulted in the release of prostacyclin into the bath solution. These results are consistent with our previous work (5) where we showed that ATP-mediated prostacyclin release from small veins was a calcium-dependent response. We further confirmed that we were measuring a metabolite of the cyclooxygenase pathway by blocking ATP-mediated prostacyclin release by 10.220.33.5 with air bubbles. Thus, the present study, we attempted to remove the endothelium by briefly perfusing the vein with an air bubble. It was surprising to us that this perturbation only reduced the ATP-stimulated prostacyclin release by ~50%. However, in a previous study of functional dilation in the hamster cremaster muscle, we showed that infusions of air into veins only reduced the functional response by ~50% (23). This raises the question of whether air bubbles are effective tools for denuding or damaging venous endothelium. In the present study, endothelial cell nuclei were identified in the histological sections of veins after perfusion with air bubbles. Thus a reduced response after an air bubble may reflect a reduced number of endothelial cells rather than complete denudation. Unfortunately, the histological techniques used in the present study did not allow for an accurate quantification of the number of endothelial cells present; thus this suggestion is entirely speculative. Alternatively, it is possible that the mechanism via which air bubbles inactivate endothelial cells is not by removal but rather by dehydration. Again, the partial attenuation of responses after air bubbles may reflect only partial damage to the endothelium.

To further examine the contribution of the endothelium to the ATP-mediated release of prostacyclin, we used deoxycholic acid to inactivate the endothelial cells. Deoxycholic acid has been reported to remove membrane proteins without actually removing the endothelial cells (26), thus rendering them inactive. Studies using deoxycholic acid have demonstrated that this compound is very effective at eliminating endothelium-dependent relaxation without altering endothelium-independent relaxation (1, 7, 8, 24). Furthermore, Cusma-Pelogia and colleagues (8) showed that inactivation of the endothelium using this substance did not alter phenylephrine- or norepinephrine-induced constriction nor did it alter the morphology of the medial smooth muscle cells. Under light microscopy we confirmed that endothelial cells were still present after
treatment with deoxycholic acid and that the smooth muscle layer was still intact. However, after the administration of deoxycholic acid, ATP-mediated 6-keto-PGF1α release was completely abolished. These studies confirm that ATP-mediated prostacyclin release from hamster epigastric veins is endothelium dependent and are consistent with studies using isolated arterial preparations (2) and cultured endothelial cells (4, 5, 20).

Throughout these experiments, we were faced with large variations in basal levels of 6-keto-PGF1α. This was particularly evident in the group of vessels used for the air perfusion. It is likely that among the other groups of vessels, variations in basal concentrations of 6-keto-PGF1α were due to differences in the number of functional endothelial cells. It would be reasonable to expect that factors such as vessel length, vessel diameter, trauma during vessel harvesting, and health of the animals may have contributed to such variations. It is difficult to attribute the very large differences observed in the air perfusion group of vessels to these factors; however, an alternative explanation is not readily available.

ATP is broken down rapidly in the circulation via the catalytic actions of ectonucleotidases in the endothelium. In the coronary circulation, ATP is broken down almost instantaneously with adenosine being one of the prominent metabolites (16). The relative contributions of ATP and adenosine to vasodilation are still controversial, and it is possible that the effect of ATP on the release of prostacyclin from veins is, in part, mediated via P1 (adenosine) receptors. To discriminate between P1 and P2 receptors, we utilized the nonselective P2 receptor antagonist reactive blue 2 (22). This antagonist effectively inhibited ATP-mediated prostacyclin release, suggesting that P2 receptors are involved in this response. Reactive blue 2 does not discriminate between P2X and P2Y receptors, and despite the large amount of evidence showing that vasodilatory endothelial P2 receptors fall into the P2Y category (22), P2X receptors may also be involved in endothelium-dependent vasodilation. P2X4 receptors have been identified on vascular endothelial cells, and activation of these receptors by ATP results in increases in intracellular calcium (27). While the results of our study do not discriminate between P2X and P2Y receptor subtypes, it is interesting to note that the refractory period, or desensitization effect after the first administration of ATP, is consistent with the involvement of a G protein-coupled receptor to which category P2Y but not P2X receptors belong.

To further support the hypothesis that ATP-stimulated prostacyclin release is mediated by P2 and not P1 receptors, we attempted to stimulate the release of prostacyclin using adenosine. Consistent with others (2, 4, 20, 25), adenosine failed to stimulate the release of prostacyclin from these veins. To ensure that the response was not mediated via AMP, which also exerts its effect via stimulation of P1 receptors, we tested the nonselective P1 antagonist XAC on ATP-stimulated prostacyclin release. This antagonist did not alter the ATP-mediated response. Only one concentration of XAC was utilized in the present study so it is possible that a higher concentration may have had an effect. However, this concentration has been shown to effectively inhibit arteriolar dilations to 100 μM adenosine and to inhibit arteriolar dilations induced by muscle contraction (6, 19). These results are consistent with those of others who have demonstrated that neither adenosine nor AMP stimulate the release of prostacyclin from endothelial cells (4, 20, 25).

The results obtained from the present study support our hypothesis that ATP stimulates the release of prostacyclin from venous endothelial cells via activation of P2Y receptors. Neither AMP nor adenosine is likely to be involved in this response; however, we cannot rule out the possibility that ADP, which, like ATP, exerts its effects via P2Y receptors, is playing a role. These results support a role for the venous endothelium in control of vascular tone.

**Perspectives**

Skeletal muscle blood flow is regulated to meet the metabolic needs of the tissue. The vasculature is arranged as a series of successively branching arterioles, all of which must dilate during periods of increased muscle metabolism. We have hypothesized that the close arteriole-venular pairing allows the arteriolar diameter to be influenced by vasoactive substances diffusing from the venules. The venular side of the circulation undergoes dramatic changes in blood chemistry during increased muscle metabolism and is thus in an optimal position to monitor the overall metabolic state of the tissue. Red blood cells can release ATP in response to changes in blood chemistry and may provide the means of communication between the metabolic state of the tissue and the vasculature. ATP-stimulated release of vasodilators, such as prostacyclin, from venules would ensure that larger arterioles located beyond the site of increased muscle metabolism and possibly beyond the distance which a conducted response can be transmitted would dilate rapidly and thus contribute to the increase in blood supply to the exercising muscle.

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ATP-MEDIATED RELEASE OF PROSTACYCLIN FROM ISOLATED VEINS